

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**

JR

Consommation  
et Corporations Canada      Consumer and  
Bureau des brevets      Patent Office  
Ottawa, Canada      (21) (A1)      2,012,311  
K1A 0C9      (22)      1990/03/15  
                    (43)      1990/09/16  
                    (52)      195-1.12  
                            C.R. CL. 530-15.14  
                                    167-103.9

5,025,5/62

(51) INTL.CL. <sup>5</sup> C12N-15/11; C12N-5/10; C07K-15/14; A61K-37/02

(19) (CA) APPLICATION FOR CANADIAN PATENT (12)

(54) New Protein-Polycation-Conjugate

(72) Beug, Hartmut - Austria ;  
Birnstiel, Max L. - Austria ;  
Cotten, Matthew - Austria ;  
Wagner, Ernst - Austria ;

(73) Boehringer Ingelheim International G.m.b.H. - Germany  
(Federal Republic of) ;

(30) (AT) A 610/89 1989/03/16

(57) 26 Claims

Notice: The specification contained herein as filed

Canada

CCA 3254 (10-89) 41

201231

1

54142J.07

"New protein-polycation conjugates"

The invention relates to the transporting of substances with an affinity for polycations, particularly nucleic acids, into the cell, the transportation being carried out by means of receptor-mediated endocytosis.

Antisense RNAs and DNAs have proved to be effective agents for selectively inhibiting certain genetic sequences in cell-free systems as well as within the living cell. Their mode of activity is based on the specific recognition of a complementary nucleic acid strand and attachment thereto, thus affecting the transcription, translation and cleaving processes. This mechanism of activity theoretically makes it possible to use antisense oligonucleotides as therapeutic agents which will block the expression of certain genes (such as deregulated oncogenes or viral genes) in vivo. It has already been shown that short antisense oligonucleotides can be imported into cells and perform their inhibiting activity therein (Zamecnik et al., 1986), even though the intracellular concentration thereof is low, partly because of their restricted uptake through the cell membrane owing to the strong negative charge of the nucleic acids.

Another method of selectively inhibiting genes consists in the application of ribozymes, i.e. RNA molecules which recognise specific RNA sequences and are able to bind to them and cleave them. Here again there is the need to guarantee the highest possible concentration of active ribozymes in the cell, for which transportation into the cell is one of the limiting factors.

In order to counteract this limiting factor, a number of solutions have already been proposed.

One of these solutions consists in direct

modification of nucleic acids, e.g. by substituting the charged phosphodiester groups with uncharged methyl phosphonates (Smith et al., 1986), carbamates (Stirchak et al., 1987) or silyl compounds (Cormier et al., 1988) or using phosphorothioates (Stern et al., 1988). Another possible method of direct modification consists in the use of nucleoside analogues (Morvan et al., 1988, Praseuth et al., 1988)).

Even though some of these proposals appear to offer a promising way of solving the problem, they do have numerous disadvantages, e.g. reduced binding to the target molecule and a reduced inhibitory effect. A chief disadvantage of the in vivo use of modified oligonucleotides is the possible toxicity of these compounds.

An alternative method to the direct modification of the oligonucleotides consists in leaving the oligonucleotide itself unchanged and providing it with a group which will impart the desired properties to it, e.g. with molecules which will make transportation into the cell easier. One of the proposed solutions within the scope of this principle consists in conjugating the oligonucleotide with polycationic compounds (Lemaitre et al., 1987).

Various techniques are known for genetic transformation of mammalian cells in vitro, but the use of these techniques in vivo is restricted (these include the introduction of DNA by means of viruses, liposomes, electroporation, microinjection, cell fusion, DEAE dextran or the calcium phosphate precipitation method).

Attempts have therefore already been made to develop a soluble system which can be used in vivo, which will convey DNA into the cells in a directed manner by means of receptor-mediated endocytosis (G.Y. Wu, C.H. Wu, 1987). This system was developed for hepatocytes and is based essentially on the following two facts:

1. On the surface of the hepatocytes there are receptors which bind specific glycoproteins and convey them into the cell.
2. DNA can be bound to polycationic compounds, e.g. polylysine, by a strong electrostatic interaction, forming soluble complexes.

The system is based on the principle of coupling polylysine with a glycoprotein of a kind to which the receptor will respond and then, by adding DNA, forming a soluble glycoprotein/polylysine/DNA complex which will be transported to the cells containing the glycoprotein receptor and, after the absorption of the complex into the cell, will make it possible for the DNA sequence to be expressed.

An object of the present invention is to provide a highly active transporting system which is more widely applicable than the known system, which can only be used for one particular cell type owing to the specific presence of the receptor on this one cell type.

According to one aspect of the invention there is provided a protein-polycation conjugate comprising a polycation bound to transferrin.

Transferrins are a class of related metal-binding transporting glycoproteins with an *in vivo* specificity for iron. Various mammalian transferrins have been identified, plasma transferrin supplying the majority of body tissue with iron. The main producer of transferrin is the liver.

Transferrin has a molecular weight of about 80,000, 6% of which consists of the sugar residues. A transferrin molecule is able to bind two molecules of iron, and this binding requires the presence of carbonate or bicarbonate ions.

The transferrin receptor, which occurs in a number

of forms which possibly deviate slightly from one another on the various cells (e.g. in the carbohydrate group) is a transmembrane glycoprotein with a molecular weight of about 180,000, whilst a receptor molecule is able to bind one or possibly two molecules of transferrin.

At the physiological pH of 7.4, the transferrin receptor has a very high affinity for  $\text{Fe}_2$  transferrin, lower affinity for the Fe transferrin and virtually no affinity for apotransferrin, even though the latter forms a very stable complex with the receptor at a pH of about 5.

Transferrin receptors have been detected in particularly large numbers in precursors of erythrocytes, placenta and liver and in measurable amounts in a number of other body tissues. A particularly interesting observation is that the receptor is highly regulated in growing cells. It has also been observed that the number of receptors is substantially increased in neoplastic tissue in vivo compared with benign lesions, indicating an increased iron requirement. The absorption mechanism of the transferrin-iron complex by receptors and the intracellular cycle thereof have been thoroughly researched.

There is still no absolute certainty as to the exact sequence of events of the release of the iron molecules by transferrin, although the majority view is that the molecules are released mainly intracellularly. In a process which is dependent on energy and temperature, Fe transferrin or  $\text{Fe}_2$  transferrin is bound to the receptor on the cell membrane. Then the complex is absorbed into a vesicle, referred to as endosome or receptosome. This is combined with another vesicle having a pH of < 5.5; the resulting acidification causes the iron to be released by the transferrin. The apotransferrin receptor complex is then transported back

to the cell membrane, where the neutral pH causes the apotransferrin to be released from the receptor into the surrounding medium. There are indications that the recycling, the functioning of which is based on the affinity of the receptor for apo- or iron transferrin, which varies at acid and neutral pH values, takes place through vesicles of the Golgi apparatus.

At a molecular level, the initiation of the transferrin cycle has not yet been explained; there are merely indications regarding some aspects, e.g. the possible role of phosphorylation (Huebers et al., 1987).

With the present invention it has been possible for the first time to make use of this active transporting system in order to convey into the cell nucleic acids, the absorption of which would otherwise be low or difficult to achieve.

The present invention includes polycation conjugates which are capable of forming complexes with substances having an affinity for polycations, particularly nucleic acids or nucleic acid analogues, and these complexes are absorbed into the cell by means of receptor-mediated endocytosis, the protein content of the conjugate being transferrin.

It has been found, surprisingly, that nucleic acids can be efficiently transported into the cell whilst maintaining their inhibitory activity, using conjugates according to the invention.

The word "transferrin" as used herein is intended to encompass both the natural transferrins and also modified transferrins which are bound by the receptor and transported into the cell (such modified transferrins may consist, for example, of a change in the amino acids or a shortening of the molecule to the fraction which is responsible for receptor binding).

The molar ratio of transferrin to polycation may, for example, be 10:1 to 1:4, although it should be borne in mind that aggregates may be formed. However, this

ratio may if necessary be within wider limits, provided that it satisfies the condition that complexing of the nucleic acid or acids takes place and it is ensured that the complex formed will be bound by the transferrin receptor and conveyed into the cell; this can easily be checked by simple experiments carried out from one case to the next.

The ratio chosen will depend particularly on the size of the polycation molecule and the number and distribution of the positively charged groupings, these criteria being matched to the size and structure of the nucleic acid or acids to be transported and to any modifications thereto. The polycations may be identical or different.

The following compounds may be used as polycations:

- a) Protamines: these are small (MW up to about 8000) strongly basic proteins, the positively charged amino acid groups of which (especially arginines) are usually arranged in groups and neutralise the negative charges of nucleic acids because of their polycationic nature (Warrant et al., 1978). The protamines which may be used within the scope of the present invention may be of natural origin or produced by recombinant methods, whilst multiple copies may be produced or modifications may be made in the molecular size and amino acid sequence. Corresponding compounds may also be chemically synthesised. When an artificial protamine is synthesised the procedure used may consist, for example, in replacing amino acid residues, which have functions in the natural protamine which are undesirable for the transporting function (e.g. the condensation of DNA) with other suitable amino acids, and/or at one end providing an amino acid (e.g. cysteine) which will enable the desired conjugation with transferrin.

- b) Histones: these are small DNA-binding proteins present in the chromatin, having a high proportion of positively charged amino acids (lysine and arginine) which enable them to bind to DNA independently of the nucleotide sequence and fold it into nucleosomes, the arginine-rich histones H3 and H4 being particularly suitable (Felsenfeld, 1978). As for the preparation and modifications thereof, the remarks made above in relation to protamines apply here as well.
- c) Synthetic polypeptides such as homologous polypeptides (polylysine, polyarginine) or heterologous polypeptides (consisting of two or more representatives of positively charged amino acids).
- d) Non-peptide cations such as polyethyleneimines.

The size of the polycations is preferably selected so that the sum of the positive charges is about 20 to 500 and this is matched to the nucleic acid which is to be transported.

The transferrin-polycation conjugates according to the invention may be produced chemically or, if the polycation is a polypeptide, by the recombinant method. Coupling by the chemical method can be carried out in a manner known *per se* for the coupling of peptides and if necessary the individual components may be provided with linker substances before the coupling reaction (this procedure is necessary when there is no functional group suitable for coupling available at the outset, such as a mercapto or alcohol group). The linker substances are bifunctional compounds which are first reacted with functional groups of the individual components, after which coupling of the modified individual components is carried out.

Depending on the desired properties of the

conjugates, particularly the desired stability thereof, coupling may be carried out by means of

- a) disulphide bridges, which can be cleaved again under reductive conditions (e.g. using succinimidyl pyridyl dithiopropionate (Jung et al., 1981)).
- b) Using compounds which are largely stable under biological conditions (e.g. thioethers, by reacting maleimido linkers with sulphydryl groups of the linker bound to the second component).
- c) Using bridges which are unstable under biological conditions, e.g. ester bonds, or using acetal or ketal bonds which are unstable under weakly acidic conditions.

The production of the conjugates according to the invention by the recombinant method offers the advantage of producing precisely defined, uniform compounds, whereas chemical coupling produces conjugate mixtures which then have to be separated.

The recombinant preparation of the conjugates according to the invention can be carried out using methods known for the production of chimeric polypeptides. The polycationic peptides may vary in terms of their size and amino acid sequence. Production by genetic engineering also has the advantage of allowing the transferrin part of the conjugate to be modified, by increasing the ability to bind to the receptor, by suitable mutations, for example, or by shortening the transferrin fraction to the part of the molecule which is responsible for the binding to the receptor. It is particularly expedient for the recombinant preparation of the conjugates according to the invention to use a vector which contains the sequence coding for the transferrin part as well as a

polylinker into which the required sequence coding for the polycationic peptide is inserted. In this way, a set of expression plasmids can be obtained, of which the plasmid containing the desired sequence can be used as necessary in order to express the conjugate according to the invention.

The nucleic acids which are to be transported into the cell may be DNAs or RNAs, with no restrictions as to the nucleotide sequence. The nucleic acids may be modified, provided that this modification does not affect the polyanionic nature of the nucleic acids; these modifications include, for example, the substitution of the phosphodiester group by phosphorothioates or the use of nucleoside analogues.

Nucleic acids which may be used within the scope of the present invention include particularly those which are to be transported into the cell for the purpose of inhibiting specific gene sequences. These include antisense oligonucleotides and ribozymes, optionally together with a carrier nucleic acid. With regard to the size of the nucleic acids the invention again permits a wide range of uses. There is no lower limit brought about by the transporting system according to the invention; any lower limit which might arise would be for reasons specific to the application, e.g. because antisense oligonucleotides with less than about 10 nucleotides would hardly be suitable owing to their lack of specificity. Using the conjugates according to the invention it is also possible to convey plasmids into the cell, the smaller plasmids which are used as carrier nucleic acids (e.g. retroviral vectors with up to 5000 bp) being of particular practical use. It is also possible to convey different nucleic acids into the cell at the same time using the conjugates according to the invention.

A further advantage of the present invention is the fact that there are polymorphisms for transferrin and

the receptor which can be exploited for the deliberate transporting of inhibiting nucleic acids into specific cells.

Within the scope of the present invention it has been possible to demonstrate that transferrin-polycation conjugates can be efficiently absorbed into living cells and internalised. The conjugates or complexes according to the invention are not harmful to cell growth. This means that they can be administered repeatedly and thus ensures a constantly high expression of genes inserted into the cell.

It has also been possible to show that the polycation-transferrin conjugates can functionally replace the native transferrin-iron complex. The fact that the transferrin-polycation/DNA complexes are absorbed through the cell by means of the transferrin receptor has been confirmed using the luciferase gene as DNA component. It has been shown that native transferrin efficiently displaces the transferrin-polycation/DNA complex, and this has been measured by the reduction in the luciferase activity in the cell.

The experiments carried out within the scope of this invention have also demonstrated that a tRNA ribozyme gene (the ribozyme being directed against a V-erbB sequence) can be introduced into erbB-transformed chicken cells using a conjugate according to the invention (polylysine-transferrin) and can attenuate the transforming activity of the oncogene. This result is all the more significant as only a small amount of ribosome gene was used in these experiments.

The ratio of nucleic acid to conjugate can vary within a wide range, and it is not absolutely necessary to neutralise all the charges of the nucleic acid. This ratio will have to be adjusted for each individual case depending on criteria such as the size and structure of the nucleic acid which is to be transported, the size of the polycation and the number and distribution of its

charges, so as to achieve a ratio of transportability and biological activity of the nucleic acid which is favourable to the particular application. This ratio can first of all be adjusted coarsely, for example by using the delay in the speed of migration of the DNA in a gel (e.g. using the mobility shift on an agarose gel) or by density gradient centrifugation. Once this provisional ratio has been obtained, it may be expedient to carry out transporting tests with the radioactively labelled complex with respect to the maximum available activity of the nucleic acid in the cell and then reduce the proportion of conjugate if necessary so that the remaining negative charges of the nucleic acid are not an obstacle to transportation into the cell.

The preparation of the transferrin-polycation/nucleic acid complexes, which are also a subject of the invention, can be carried out using methods known per se for the complexing of polyionic compounds. One possible way of avoiding uncontrolled aggregation or precipitation is to mix the two components together first of all at a high (about 1 molar) concentration of common salt and subsequently to adjust to physiological saline concentration by dialysis or dilution. Preferably, the concentrations of DNA and conjugate used in the complex forming reaction are not too high (more than 100 µg/ml), to ensure that the complexes are not precipitated.

The preferred nucleic acid component of the transferrin-polycation-nucleic acid complex according to the invention is antisense-DNA, antisense-RNA or a ribozyme or the gene coding for it. When ribozymes and antisense-RNAs are used it is particularly advantageous to use the genes coding for these RNAs which inhibit the function of RNA, preferably together with a carrier gene. By introducing the gene into the cell, a substantial amplification of the RNA is ensured, as against the importing of RNA as such, and consequently a

sufficient amount to inhibit the biological reaction is ensured. Particularly suitable carrier genes are the transcription units, e.g. tRNA genes, required for transcription by polymerase III. Ribozyme genes, for example, may be inserted into them in such a way that when the transcription is carried out the ribozyme is part of a compact polymerase III transcript. Using the transporting system according to the present invention it is possible to intensify the activity of these genetic units, by guaranteeing an increased initial concentration of the gene in the cell.

The invention further relates to a process for introducing nucleic acid or acids into human or animal cells, preferably forming a complex which is soluble under physiological conditions.

The invention further relates to pharmaceutical preparations containing as the active component a nucleic acid which specifically inhibits a gene, complexed with a transferrin-polycation conjugate, e.g. in the form of a lyophilisate. Such pharmaceutical preparations may be used to inhibit pathogenic viruses such as HIV or related retroviruses, oncogenes or other key genes which control growth and/or differentiation of cells, e.g. the c-fos gene or the c-myc gene, together with antisense oligonucleotides, antisense oligonucleotide analogues or ribozymes or the DNAs coding for them, optionally together with a carrier nucleic acid, in humans or animals.

Another field of use is in fighting diseases by inhibiting the production of undesirable gene products, e.g. the major plaque protein which occurs in Alzheimer's disease or proteins which cause autoimmune diseases.

The invention will now be illustrated by way of the following non-limiting Examples with reference to the drawings in which:-

Figure 1 is a graph of absorption at 280 nm of material eluted from an ion exchange column (Example 1c);

Figures 2 to 4 are photographs of stained electrophoretic gels (Examples 1c, 3c and 4);

Figure 5 comprises photomicrographs of cells under fluorescent light (Example 5);

Figures 6A and B are graphs showing haemoglobin levels against days in culture and concentration of an iron-bearing substance respectively (Example 7);

Figure 7 shows nucleic acid sequences insertable into a plasmid;

Figures 8 and 9 are graphs showing light emitted from cells versus  $\mu\text{g}$  DNA;

Figures 10A and 10B are graphs showing light emitted from cells with respect to the presence of certain polycations/polycation-conjugate;

Figure 11 illustrates three mRNA sequences;

Figure 12 is a photograph of an electrophoretic gel;

Figure 13 is a graph showing the relationship of bound substance to total substance as described in Example 11;

Figures 14 and 15 are graphs of cell fluorescence as described in Examples 12 and 13.

#### Example 1

##### Preparation of transferrin-polylysine 90 conjugates

Coupling was carried out using methods known from the literature (Jung et al., (1981)) by introducing disulphide bridges after modification with succinimidyl-pyridyl dithiopropionate.

a) 3-(2-Pyridyl)dithio)propionate-modified transferrin:

6 ml of a solution, gel-filtered over Sephadex G-25, of 120 mg (1.5  $\mu$ mol) of transferrin (from chicken albumin, Sigma, Conalbumin Type I, iron-free) in 3 ml of 0.1 M sodium phosphate buffer (pH 7.8) were mixed with 200  $\mu$ l of 15 mM ethanolic solution of succinimidyl 3-(2-pyridyl)dithio)propionate (3  $\mu$ M, SPDP, Pharmacia) with vigorous shaking and the mixture was left to react for 1 hour at ambient temperature with occasional shaking. Low molecular reaction products and traces of reagent were removed using a gel column (Sephadex G-25, 14 x 180 mm, 0.1 M sodium phosphate buffer pH 7.8) and 7 ml of the product fraction were obtained; the content of pyridyl dithiopropionate residues bound to transferrin was determined by means of one aliquot, after reduction with dithiothreitol, by photometric measurement of the quantity of pyridin-2-thione released and the result was 2.6  $\mu$ mol. Human transferrin (Sigma, iron-free) was modified in exactly the same way.

b) Mercaptopropionate-modified polylysine 90 (pL 90):

A solution of 18 mg (about 1.0  $\mu$ mol) of poly(L)lysine-hydrobromide (Sigma, fluoresceinisothiocyanate (= FITC)-labelled, molecular weight of about 18,000 - corresponding to an average degree of polymerisation of about 90) in 3 ml of 0.1 M sodium phosphate (pH 7.8) was filtered over Sephadex G-25 (the fluorescent labelling was carried out in sodium bicarbonate buffer pH 9 for 3 hours). The polylysine solution was diluted with water to 7 ml, combined with 270  $\mu$ l of a 15 mM ethanolic solution of SPDP with thorough shaking and then left to react for 1 hour in the dark at ambient temperature and with occasional shaking. After the addition of 0.5 ml of 1 M sodium acetate buffer (pH 5.0) the mixture was filtered over

Sephadex G-25 to separate off any lower molecular substances (eluant: 20 mM sodium acetate buffer pH 5.0). The product fraction (ninhydrin staining, fluorescence) was evaporated down in vacuo, adjusted to pH about 7 with buffer, a solution of 23 mg (150  $\mu$ mol) of dithiothreitol in 200  $\mu$ l of water was added and the mixture was left to stand for 1 hour in the dark under argon at ambient temperature. Excess reducing agent was separated off by further gel filtration (Sephadex G-25, 14 x 130 mm column, 10 mM sodium acetate buffer pH 5.0) and 3.5 ml of product solution of fluorescently labelled polylysine were obtained, containing 3.8  $\mu$ mol of mercapto groups (photometric determination using Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoic acid)).

c) Transferrin-polylysine conjugates:

The solution of modified transferrin obtained in a) (7 ml in 0.1 M sodium phosphate buffer pH 7.8, about 1.5  $\mu$ mol transferrin with about 2.6  $\mu$ mol pyridyl dithiopropionate residues) was rinsed with argon; 2.0 ml of the solution of mercapto-modified polylysine obtained in b) (in 10 mM sodium acetate buffer pH 5.0, corresponding to about 0.6  $\mu$ mol of polylysine with about 2.2  $\mu$ mol of mercapto groups) were added, the mixture was rinsed with argon, shaken and left to react for 18 hours at ambient temperature in the dark and under argon. The reaction mixture was diluted with water to 14 ml and separated by ion exchange chromatography (Pharmacia Mono S column HR 10/10, gradient elution, buffer A: 50 mM HEPES pH 7.9, buffer B: A + 3 M sodium chloride, 0.5 ml/min, Fig. 1). Non-conjugated transferrin was eluted at the start, product fractions at about 0.66 - 1.5 M sodium chloride.

Averaged over all the fractions, conjugates were obtained containing a ratio of transferrin to polylysine of 1.3:1.

The conjugated products (ninhydrin staining, in UV at 280 nm protein absorption, and fluorescence measurement of FITC-labelled polylysine at 495 nm) were collected in 6 fractions each containing about 10 mg of transferrin. The fractions were first dialysed against a 100 mM iron(III)citrate solution (adjusted to pH 7.8 with sodium hydrogen carbonate) and then twice more with 1 mM HEPES buffer (pH 7.5).

Sodium dodecylsulphate gel electrophoresis (10% SDS, 8% polyacrylamide), see Fig. 2, showed an approximately equal content of transferrin in all 6 fractions after pretreatment with 2-mercaptoethanol (Fig. 2A), whereas in the unreduced samples there were no visible bands for free transferrin, only less widely migrating conjugates (Fig. 2B, T = untreated transferrin; 1-6 = conjugate fractions 1-6).

Example 2

Preparation of transferrin-polylysine 270 and transferrin-polylysine 450 conjugates (pL270 and pL450)

- a) Modified transferrin was produced analogously to Example 1 a)
- b) Preparation of modified polylysine 270 and polylysine 450

A gel-filtered solution of 0.33  $\mu\text{mol}$  polylysine 270 (with an average degree of polymerisation of 270 lysine groups, with or without fluorescent labelling; corresponding to 19 mg of hydrobromide salt) in 1.2 ml of 75 mM sodium acetate buffer was adjusted to pH 8.5 by the addition of sodium carbonate buffer. 182  $\mu\text{l}$  of a 15 mM ethanolic solution of SPDP (1.9  $\mu\text{mol}$ ) was added with vigorous stirring. One hour later, 200  $\mu\text{l}$  1 M sodium acetate pH 5 were added; after gel filtration

with 20 mM sodium acetate, a solution was obtained containing 0.27  $\mu$ mol of polylysine 270 with 1.3  $\mu$ mol mercapto groups (4.8 linkers per polylysine chain). Analogously, 0.20  $\mu$ mol of polylysine 450 (with an average degree of polymerisation of 450 lysine groups) were modified with 2.25  $\mu$ mol of SPDP, obtaining a product of 0.19  $\mu$ mol polylysine 450 with 2.1  $\mu$ mol mercapto groups (11 linkers per polylysine chain). Analogously to Example 1 b), the dithiopyridine groups were reduced with dithiothreitol, in order to obtain the free sulfhydryl components.

c) Preparation of transferrin-polylysine conjugates

Transferrin-polylysine 270 conjugates were prepared by mixing 1.0  $\mu$ mol of modified transferrin in 100 mM phosphate buffer, pH 7.8, with 0.14  $\mu$ mol of modified polylysine 270 (in 20 mM sodium acetate buffer) with the exclusion of oxygen in an argon atmosphere. After 18 hours at ambient temperature the reaction mixture was diluted with water to a volume of 10 ml and fractionated by cation exchange chromatography (Pharmacia Mono S column HR 10/10; gradient elution, buffer A: 50 mM HEPES pH 7.9; buffer B: A + 3 M sodium chloride; UV absorption at 280 nm and fluorescence measurement, excitation 480 nm, emission 530 nm). The excess of non-coupled transferrin was eluted first; the product fractions were eluted at between 30% and 50% gradient B and pooled in 3 conjugate fractions (molar ratios of transferrin to polylysine: pool A: 5.5 to 1; pool B: 3.4 to 1; pool C: 1.8 to 1). The conjugates were obtained in an average yield of 0.23  $\mu$ mol transferrin with 0.075  $\mu$ mol of polylysine 270.

Transferrin-polylysine 450 conjugates were prepared in a similar manner, starting from 1.2  $\mu$ mol of modified transferrin according to Example 1 a) (in 20 mM HEPES pH 7.9 containing 80 mM sodium chloride) and 71 nmol of

mercapto-modified polylysine 450 according to Example 2 b) in acetate buffer.

The reaction mixture was purified by gel permeation chromatography (Pharmacia Superose 12 column, 1 M guanidine chloride pH 7.3) and after dialysis (20 mM HEPES pH 7.3, containing 100 mM sodium chloride) yielded transferrin-polylysine-conjugates containing 0.40  $\mu$ mol of transferrin with 38 nmol of polylysine 450.

Iron was incorporated by adding 6 - 12  $\mu$ l of 100 mM iron citrate buffer (containing sodium bicarbonate adjusted to pH 7.8) to the samples, per mg of transferrin fraction.

Example 3

a) Preparation of transferrin-protamine conjugates

Modified transferrin was prepared as in Example 1 a).

b) Preparation of 3-mercaptopropionate-modified protamine

To a solution of 20 mg (3  $\mu$ mol) of protamine trifluoracetate salt (prepared by ion exchange chromatography from salmon protamine (= salmin)-sulphate, Sigma) in 2 ml of DMSO and 0.4 ml of isopropanol, containing 2.6  $\mu$ l (15  $\mu$ mol) of ethyl diisopropylamine, was added a solution of 30  $\mu$ mol of SPDP in 250  $\mu$ l of isopropanol and 250  $\mu$ l of DMSO in several batches over a period of one hour. After 3.5 hours at ambient temperature the solution was evaporated down under high vacuum and taken up in 0.5% acetic acid containing 10% methanol. Gel filtration (Sephadex G10; 0.5% acetic acid with 10% methanol) yielded, after lyophilisation, 16 mg (2.5  $\mu$ mol of protamine acetate salt, modified with 2.5  $\mu$ mol of dithiopyridine linker.

Reduction of 1.75  $\mu\text{mol}$  of protamine (containing 1.75  $\mu\text{mol}$  of linker) with 16 mg of dithiothreitol in sodium bicarbonate buffer, pH 7.5, for 1 hour under argon, followed by adjustment of the pH to 5.2 and gel filtration over Sephadex G10 with 20 mM sodium acetate buffer, pH 5.2, yielded a protamine solution modified with 1.6  $\mu\text{mol}$  of mercaptopropionate linker.

c) Preparation of transferrin-protamine conjugates

The reaction of the protamine solution obtained in b) (1.6  $\mu\text{mol}$  linker) with 1.34  $\mu\text{mol}$  of transferrin (modified with 3.1  $\mu\text{mol}$  of dithiopyridine linker) and subsequent purification by cation exchange chromatography as described for transferrin-polylysine conjugates, yielded four product fractions A - D eluted one after the other, containing 90, 320, 240 and 120 nMol, respectively, of modified transferrin with increasing amounts of protamine (determined by SDS gel electrophoresis; 10% SDS, 8% polyacrylamide, Coomassie blue staining). Fig. 3 shows the results of the SDS gel electrophoresis. The Tfprot conjugate fractions A - D more slowly migrating bands (a), whereas in  $\beta$ -mercaptoethanol-reduced samples (b) only the transferrin band was visible. Dialysis and the incorporation of iron were carried out as described for the transferrin-polylysine conjugates TfpL270 and TfpL450 in Example 2.

Example 4

Preparation of complexes of transferrin-polycation conjugates with DNA

The complexes were prepared by mixing dilute solutions of DNA (30  $\mu\text{g}/\text{ml}$  or less) with the transferrin-polycation conjugates. In order to prevent precipitation of the DNA complexes, phosphate-free

buffer was used (phosphates reduce the solubility of the conjugates). The binding of the DNA to the polycation conjugates under physiological ionic conditions was confirmed by a gel mobility shift assay using lambda DNA  $^{32}\text{P}$ -labelled at the 3'-end, cut with EcoR 1/Hind III (Fig. 4). To each sample of 1  $\mu\text{l}$  (35 ng) of DNA were added 3  $\mu\text{ml}$  of a 100 mM HEPES buffer pH 7.9, containing 1 M sodium chloride, and the samples were mixed with increasing amounts (10 ng to 1000 ng) of transferrin conjugates in 11  $\mu\text{l}$  of aqueous solution, resulting in a final concentration of sodium chloride of 200 mM. Electrophoresis on a 1% agarose gel with 1 x TAE eluting buffer was carried out at 50 Volts (45 mA) for 2.5 hours; the gel was dried, followed by autoradiography for 2 hours at -80°C using an XAR film (Kodak).

Example 5

Transporting of transferrin-polylysine conjugates into living cells

In order to demonstrate that the transferrin-polylysine conjugates described in Example 1 are efficiently absorbed into living erythroblasts, FITC-labelled conjugates were used. It is known (Schmidt et al, 1986) that FITC-labelled transferrin was detectable in vesicles inside the cell (when examined under a fluorescence microscope) after some hours' incubation with erythroblasts from which transferrin had previously been removed.

In the present Example, erythroblasts (transformed by an EGF-receptor retrovirus, Khazaie et al, 1988) were incubated for 18 hours in a transferrin-free differentiating medium (composition in Zenke et al, 1988) at 37°C (cell concentration  $1.5 \times 10^6/\text{ml}$ ). After addition of the various transferrin-polylysine conjugates (or, as a control, the corresponding amount

conjugates (or, as a control, the corresponding amount of sterile twice distilled water), the cells were incubated at 37°C in the presence of 10 ng/ml EGF in order to maintain the transformed state). After 24 and 48 hours, about  $5 \times 10^5$  cells were removed, washed once in phosphate-buffered physiological saline (PBS; pH 7.2), fixed with 50 times the volume of a mixture of 3.7% formaldehyde and 0.02% glutaraldehyde in PBS (10 minutes, 40°C), washed once in PBS, embedded in Elvanol and examined under a fluorescence microscope (Zeiss Axiophot, Narrow Band FITC and TRITC activation). At the same time, the growth rate of the cells was determined in other aliquots of the various mixtures. 100  $\mu$ l cell suspension were taken and the incorporation of  $^3\text{H}$ -thymidine (8  $\mu\text{Ci}/\text{ml}$ , 2 hours) was determined as described in Leutz et al, 1984. Fig. 5 shows that the erythroblasts incubated with transferrin-polylysine show 2 to 10 strongly fluorescing vesicles after 24 hours, which cannot be detected in the controls. Table A shows that, with the exception of fraction 6, all the conjugates have been absorbed by virtually all the cells.

Fig. 5 shows fluorescence images of chicken erythroblasts which have been incubated for 24 hours without (A) or with FITC-labelled transferrin-polylysine conjugates (B,C). When they are activated with blue light (B, in order to detect FITC), significantly more fluorescing vesicles can be detected in each cell. The specificity of this fluorescence is shown by the fact that the vesicle fluorescence does not occur when activated with green light (C: at which a similar non-specific fluorescence of the cells can be seen as in A) (C).

The fact that the cells grow equally rapidly in all the samples (as measured by the incorporation of tritiated thymidine ( $^3\text{H TdR}$ ), cf Table A at the end of the description) shows that the cells are not

dimensioned by the polylysine constructs and consequently non-specific uptake (e.g. through cell membranes which have become permeable) can be ruled out.

Example 6

The objective of the tests carried out in this Example was to show that the transferrin-polylysine conjugates used here are used by the cell in the same way as native transferrin, i.e. they pass through the normal transferrin cycle with similar efficiency. Erythroblasts which can be induced to mature into normal erythrocytes by "switching off" the transforming oncogene are particularly suitable as a test system for this purpose (Beug et al, 1982). The literature shows that for normal maturation such cells require high concentrations of transferrin-iron complex (100 to 200 µg/ml, 3 times lower concentrations prevent the cells from maturing and will result in the death of the cells after several days (Kowenz et al, 1986)). It has also been shown (Schmidt et al 1986) that recycling, i.e. the reuse of transferrin receptors and hence a transferrin cycle proceeding at optimum speed are indispensable for normal *in vitro* differentiation.

Erythroblasts (transformed by the EGF-receptor retrovirus) were induced to differentiate by the removal of EGF and the addition of an optimum amount of partially purified chicken erythropoietin (Kowenz et al., 1986, free from transferrin). Incubation was carried out at a cell concentration of  $1 \times 10^6$ /ml in transferrin-free differentiating medium at 42°C and 5% CO<sub>2</sub>. At the start of incubation, either native transferrin-iron complex (Sigma, 100 µg/ml) was added or the iron-saturated transferrin-polylysine conjugates were added (concentration again 100 µg/ml). The growth and maturity of the cells were analysed at 24 and 48 hours by the following methods:

1. by determining the number of cells (using a Coulter Counter, Model ZM, Beug et al, 1984)
2. by recording cell size distributions (using a Coulter Channelyzer Model 256) and
3. by photometric determination of the haemoglobin content of the cells (Kowenz et al., 1986).

In addition, aliquots of the mixtures were centrifuged after 72 hours in a cytocentrifuge (Shandon) on an object carrier and subjected to histochemical investigation to detect haemoglobin (staining with neutral benzidine plus Diff-Quik rapid staining for blood cells, Beug et al 1982).

The results in Table B at the end of the description clearly show that cells which were induced to differentiate in the presence of the polylysine-transferrin conjugates fractions 1 to 5 mature just as efficiently and as fast as those which were incubated with native transferrin-iron. The cells in the transferrin-free controls, on the other hand, showed a much slower cell growth and accumulated only small amounts of haemoglobin. Investigation of cell phenotype on stained cytopsin preparations showed that the cells incubated with polylysine-transferrin conjugates were matured into late reticulocytes (late reticulocytes, Beug et al., 1982) in just the same way as those which had been treated with native transferrin, whereas the cells incubated without transferrin constituted a mixture of disintegrated and immature cells resembling erythroblasts (Schmidt et al, 1986). Only the cells treated with transferrin-polylysine fraction 6 showed a lower haemoglobin content and a higher percentage of immature cells (Table B). This shows that fraction 6 conjugated with a particularly large amount of polylysine operates less well in the transferrin cycle. At the same time, this result indicates the sensitivity of the test method.

Example 7

Just as in Example 6, various transferrin-polylysine conjugates and transferrin-protamine conjugates were examined for their ability to functionally replace the native transferrin-iron complex in the maturation of chicken erythroblasts into erythrocytes.

It has already been shown that terminally differentiating chicken erythroblasts demand an optimally functioning transferrin cycle; i.e. without transferrin or if the transferrin receptor recycling is inhibited, the cells die off (Kowenz et al., 1986; Schmidt et al., 1986). Since the partially purified chicken erythropoietin normally used still contains transferrin, EPO was replaced by a transferrin-free, partially purified erythroid growth factor in order to permit erythroid differentiation (REV factor; Kowenz et al., 1986; Beug et al., 1982): as target cells, erythroblasts which had been transformed with a retrovirus containing the human epidermal growth factor receptor (EGFR) together with a temperature-sensitive v-myb oncogen were replicated in CFU-E medium (Radke et al., 1982) in the presence of 20 ng/ml of EGF. These cells are activated to replicate abnormally by EGF, whilst the withdrawal of the growth factor EGF and the simultaneous addition of REV factor causes the cells to enter into normal differentiation. After being washed twice in transferrin-free medium the cells were transferred into transferrin-free medium and varying quantities of iron-saturated transferrin or transferrin-polycation conjugates were added (before or after being complexed with plasmid DNA). After 1, 2 and 3 days' incubation at 42°C the differentiating state of the cells was determined by cytocentrifugation and histochemical staining or by quantitative haemoglobin measurement.

The results of these tests are shown in Fig. 6 or Table C (which is at the end of the description).

The cells ( $1 \times 10^6/\text{ml}$ ) were added to conalbumin-free differentiating medium (Zenke et al., 1988), supplemented by 1  $\mu\text{g}/\text{ml}$  of insulin and REV factor at an optimum concentration (Kowenz et al., 1986; dilution 1:5,000), once without additives (triangles), once with iron-saturated conalbumin (circles) and once with iron-saturated Tfpl 270 conjugates (squares) (100  $\mu\text{g}/\text{ml}$  in each case); in 14 mm dishes. After incubation for 24 and 48 hours, the haemoglobin content was photometrically determined in 100  $\mu\text{l}$  aliquots. The shaded area shows the haemoglobin content of cells grown without transferrin (average from 4 measurements; Fig. 6A).

In order to analyse the erythroid differentiation as a function of the concentration of transferrin or transferrin-polylysine, the cells were placed in medium containing the specified amounts of iron-saturated conalbumin (open circles), Tfpl 90 (open squares) or Tfpl 270 (solid squares) as described above and after 2 days the haemoglobin content was determined photometrically (Fig. 6B).

Table C:

The erythroid differentiation was monitored by photometric haemoglobin measurement (see Fig. 6), by counting in a Coulter counter or by cytocentrifugation and subsequent neutral benzidine staining (to determine the haemoglobin) plus histological dyes (Diff Quik; Beug et al., 1982b). The final concentrations of transferrin and transferrin conjugates in test 1 were 60  $\mu\text{g}/\text{ml}$ ; in tests 2 and 3 they were 100  $\mu\text{g}/\text{ml}$ . The DNA concentration in test 2 was 10  $\mu\text{g}/\text{ml}$ . The proportion of disintegrated cells, mature cells (LR: late reticulocytes; E: erythrocytes) and immature cells (Ebl)

was determined using the methods described by Beug et al., 1982b and Schmidt et al., 1986. The results obtained show that two different transferrin-polylysine conjugates (TfpL90 or TfpL270) as well as the transferrin-protamine conjugate are capable of functionally replacing native transferrin, by ensuring the rapid transfer of iron into differentiating red cells, the specific activity thereof being 1.5 to 2 times lower (cf. Fig. 6). The complexing of DNA with transferrin-polylysine 270 and transferrin protein did not materially alter the biological activity of the conjugates. In a control experiment it was established that, when polylysine or protamine is added, mixed with a suitable quantity of iron citrate instead of the transferrin conjugates, the cells were enable to differentiate and died off, just like the cells in the comparison samples which had been incubated without transferrin.

All in all, the tests according to Examples 6 and 7 have shown that both types of polycation-transferrin conjugates transported iron only slightly less efficiently than natural transferrin.

Example 8

Polylysine-transferrin conjugates make it possible for DNA to be absorbed into chicken erythroblasts.

The present Example was intended to investigate whether DNA of a size corresponding to that of tDNA ribozymes (see Fig. 7) is capable of being efficiently transported into the interior of the cell by transferrin-polylysine conjugates. In the present Example, tDNA with an insert of the sequence

CGTTAACAAAGCTAACGTTGAGGGGCATGATATCGGGCC  
CCGGGCAATTGTCGATTGCAACTCCCCGTACTATAGC

molecular weight about 300,000 was used, terminally labelled with gamma 32P ATP (Maniatis). About 0.3 µg of this DNA, dissolved in 20 µl of TE buffer were mixed either with 10 µg of native transferrin, with 10 µg of transferrin-polylysine conjugate fraction 3, in each case dissolved in 50 µl of twice distilled water plus 400 µg/ml of bovine serum albumin (Beug H., et al., 1982) or with 50 µl of this solvent without transferrin. The DNA protein mixtures were each added to 2 ml of transferrin-free differentiating medium, 4x10<sup>6</sup> chicken erythroblasts were added (which had previously been transformed with an EGF receptor retrovirus and pre-incubated for 18 hours in transferrin-free medium in the presence of EGF (Khazaie K., et al., 1988) and the mixtures were incubated for 8 hours at 37°C and 5% CO<sub>2</sub>. Then the cells were centrifuged off, the supernatant was removed and the cells were washed 3 times in transferrin-free medium. Cell sediment and culture medium were taken up in 1% SDS, 1 mg/ml of proteinase K, 300 mM NaCl, 20 mM tris pH 8.0, 10 mM EDTA (PK/SDS buffer), incubated for 30 minutes at 37°C, extracted with phenol/chloroform, and the DNA isolated by ethanol precipitation. Isolated DNA with a radioactivity of 2000 cpm in all were separated on a non-denaturing 3.5% acrylamide gel (TBE, Maniatis) and the DNA was detected by autoradiography. It was shown that, in the cell sample treated with transferrin-polylysine, approximately 5 to 10 times more DNA had been absorbed by the cells than in the control samples with native transferrin.

Example 9

Polylysine-transferrin conjugates make it possible for plasmid-DNA to be absorbed into and expressed in chicken erythroblasts.

In these tests, plasmid-DNA containing the Photinus pyralis luciferase gene as reporter gene was used to investigate gene transfer and expression. For this purpose, pRSVluc plasmid DNA (De Wet, J.R., et al., 1987) was prepared using the Triton-X lysis standard method (Maniatis), followed by CsCl/EtBr equilibrium density gradient centrifugation, decolorising with butanol-1 and dialysis with 10 mM Tris/HCl, pH 7.5, 1 mM EDTA. In a typical complex forming reaction, 10 µg of transferrin-polylysine or transferrin-protamine conjugates were slowly added, with careful stirring, to 3 µg of pRSVluc plasmid DNA contained in 250 µl of 0.3 M NaCl (it was established that these conditions are adhered to, up to 100 µg of transferrin-polycation conjugate and 30 µg of plasmid-DNA can be used in a final volume of 500 µl without precipitation of the conjugate/DNA complexes). After 30 minutes at ambient temperature, the complexes were added directly to 5 - 10 x 10<sup>6</sup> HD3 cells (0.5 - 1 x 10<sup>6</sup> cells per ml, EBM+H medium (Beug et al., 1982a; 37°C, 5% CO<sub>2</sub>) and the mixtures were incubated for 16 to 48 hours (the cell line used was the ts-v-erbB transformed chicken erythroblast cell line HD3). The cells were harvested (5 min at 1500 x g, 4°C, washed twice with phosphate-buffered saline (PBS) and taken up in 100 µl of 0.25 M tris/HCl, pH 7.5. Cell extracts were prepared by three cycles of freezing and thawing, followed by high-speed centrifugation (15 min, 18,500 x g, 4°C). Aliquots of these cell extracts were examined for the presence of luciferase enzyme activity (De Wet, J.R., et al., 1987). The bioluminescence was measured using clinilumate (Berthold, Wildbach, BRD). It was established that the presence of the transferrin-polycation/DNA complexes in the culture medium does not have any harmful effects on cell growth or replication. As can be seen from Fig. 8, maximum luciferase activity was achieved when using 3 µg of DNA/10 µg of Tfpl and 0.3 - 1 µg of DNA/Tfprot.

Assuming that all the conjugate/DNA complexes formed were identical, this corresponds to a molar ratio of 25 or 75 conjugate molecules per molecule of plasmid DNA. It can be concluded from this that the DNA in the complex is entirely covered by the conjugate molecules, and indeed at a conjugate/DNA ratio which obviously guarantees electroneutrality (calculated on the basis of the positive charges in the polycation which are necessary in order to neutralise the negative charges of the phosphate groups in the DNA). This assumption agrees with the observation that, compared with transferrin-polylysine, three times more transferrin/protamine, less strongly positively charged, is required for optimum complex forming and gene transfer. This assumption also accords with the results for the conjugate/DNA ratio obtained in Example 4 required for efficient complex formation.

The sensitivity of this gene transfer system was determined using a Tfpl/DNA ratio which had been adjusted to the optimum for complex forming. The results of these tests are shown in Fig. 9: less than 1 ng of plasmid DNA coding for luciferase still shows a detectable signal. The use of more than 2  $\mu$ g of plasmid DNA, complexed with 6  $\mu$ g of Tfpl or 20  $\mu$ g of Tfprot, does not result in any further increase in luciferase activity, presumably because the system is saturated. It was also found that no special concentration of salt or ions is needed for the complex forming, since Tfpl/DNA complexes formed at various salt concentrations (0, 20, 50, 100, 200 mM NaCl) prove to be equally effective in gene transfer experiments. (Fig. 8 and Fig. 9: circles indicate Tfprot, squares indicate Tfpl). It was possible to demonstrate that the absorption of transferrin-polycation/DNA complexes into the cells was effected via the transferrin receptor. First of all, as illustrated in Fig. 10A, it was found that the luciferase activity achieved by Tfpl-DNA complexes is at

least 100 times higher than the activity measured for pL-DNA complexes. A comparison test shows that a mixture of polylysine and transferrin alone did not facilitate the uptake of plasmid DNA. In another test, an excess of native transferrin was added to a constant quantity of Tfpl-DNA complex. Fig. 10B shows that free transferrin in the medium efficiently competes for the DNA uptake mediated by Tfpl, resulting in a reduction in the luciferase enzyme activity. From this it can be concluded that the uptake of the Tfpl-DNA complexes by the cell is effected via the transferrin receptor.

Example 10

In preliminary tests it was established, by the transfection of chicken fibroblasts with erbB cut DNA that the erbB cut ribozyme-tDNA is expressed in chicken cells.

This Example shows that tDNA ribozymes directed against the erbB oncogene can be introduced into erbB-transformed chicken erythroblasts with the aid of polylysine-transferrin conjugates and can weaken the transforming activity of the oncogene.

Two tRNA ribozyme genes, directed against the translation initiation region of erbB, were constructed (see Figs. 7 and 11). About 100 µg of each plasmid containing the gene were digested in EcoRI in order to free the tRNA ribozyme gene on a 325 bp fragment.

The digestion products were terminally labelled by means of klenow fragment and purified by gel electrophoresis using a 2% agarose/TBE gel. The vector fragment and the tRNA-ribozyme gene fragments were located by staining with ethidium bromide, cut out and recovered by electroelution, extraction with phenol/chloroform and chloroform and ethanol precipitation. The purified, radioactively labelled DNA fragments were then used, with the aid of the

transferrin-polylysine transporting system, to determine the uptake and inhibition of the erbB-RNA. The vector pSPT 18 was used as the control DNA.

The test cell system used was a chicken erythroblast cell line transformed by a temperature-sensitive mutant (ts 34, Graf et al. 1978) of the avine erythroblastosis virus AEV (Beug et al, 1982 b). The erbA oncogene which is also expressed in these cells can be inhibited by a specific protein kinase inhibitor (H 7). (It was found that the v-erbA oncogene is phosphorylated in vivo and in vitro (i.e. as a bacterially expressed protein) at two sites, namely Ser 28 and Ser29, by protein kinase C or by cAMP-dependent protein kinase. Mutation of these serines to form alanines prevents phosphorylation and destroys the v-erbA oncogene activity. H7 is a specific inhibitor of these two kinases and is capable of selectively stopping the changes caused by v-erbA (e.g. blocking of differentiation) in erythroblasts which contain v-erbA-v-erbB.)

It is known that erythroblasts in which the erbB oncogene is inactivated - e.g. by increasing the temperature in the case of a temperature-sensitive erbB mutant, are induced to mature into erythrocytes. One of the first indications of this process is the induction of haemoglobin synthesis, which can be detected by a sensitive test (acidic benzidine staining, Orkin et al, 1975, Graf et al, 1978) at the level of the single cells. Thus, a specific increase in the number of benzidine-positive cells might be expected, as a phenotypical effect of a ribozyme directed against erbB in this test system.

The test series on which this Example is based was carried out as follows: the various DNA preparations (see above and Table D), dissolved in 30 µl of TE buffer, were mixed with 10 µg of native transferrin-iron complex or transferrin-polylysine conjugate (dissolved

in 50 µl of twice distilled water) and then incubated for 30 minutes at 37°C.

In the case of the vector DNA (10 µg), correspondingly more (100 µg) of the transferrin preparations was used. The DNA transferrin-DNA mixtures were added to 1 ml of transferrin-free differentiating medium (Zenke et al., 1988). The test cells (per batch  $3 \times 10^6$ ) were incubated before the test for 60 minutes in transferrin-free differentiating medium at 42°C (to intensify the uptake of transferrin) and added to the DNA-transferrin-containing mixtures. After 6 hours, 18 hours and 68 hours (see below for treatment of cells), samples were taken as described, separated into supernatant and cell sediment, taken up in PK/SDS buffer and the DNA was analysed.

Fig. 12 shows that, analogously to Example 8, in the cell sample treated with transferrin-polylysine, about 5-10 times more DNA was absorbed by the cells than in the control samples with native transferrin.

Trace m: molecular weight marker: pBR322 DNA, cleaved with HpaII and radioactively labelled using the klenow fragment of DNA polymerase with alpha- $^{32}$ P-CTP (Maniatis)

Trace 1: 2000 cpm ES13 fragment

Trace 2: material from cells treated with transferrin and ES13

Trace 3: material from cells treated with transferrin-polylysine and ES13

After the end of incubation (6 hours) the cells were centrifuged off and incubated in transferrin-containing differentiating medium with erythropoietin and insulin (Kowenz et al, 1986, Zenke et al 1988, 2 ml per batch and at 37°C, i.e. in the presence of an active v-erbB protein) for a further 72 hours.

The following results were obtained:

1. As in Example 8, an increased uptake of DNA could be observed in the size of the erbB cut DNAs in the cell samples treated with transferrin-polylysine (about a 5-fold increase).
2. Table D shows that in every case where erbB cut ribozyme tDNA was introduced into erbB transformed erythroblasts with the aid of polylysine-transferrin constructs, the percentage of benzidine-positive cells was significantly increased (approximately doubled) (the standard used was the samples treated with vector DNA in which the use of polylysine-transferrin conjugates, as expected, did not lead to any increase in the number of benzidine-positive cells).

Example 11

Efficient binding and internalisation of transferrin-polylysine/DNA complexes in haematopoietic chicken cells

The binding of TfpL and TfpL-DNA to cell surface receptors was measured with tritium-labelled substances using the method described by Stein et al., 1984.  $^3\text{H}$ -labelled TfpL was prepared by conjugation of labelled polylysine with transferrin using the method described in Example 1. The labelling of polylysine 90 was carried out by treating with formaldehyde and  $^3\text{H}$ -labelled sodium borohydride (Ascoli and Puet, 1974). The results of these tests are shown in Fig. 13. Labelled TfpL 90 (squares) or labelled TfpL 90 complexed with pB-SK- DNA (Promega Biotech, prepared by Triton-X lysis, CsCl/EtBr equilibrium density gradient centrifugation, decolorisation with 1-butanol and dialysis against 10 mM Tris/HCl pH 7.5, 1 mM EDTA) (triangles) were investigated for their specific binding to the

transferrin receptor of HD3 cells. For this purpose the conjugates or complexes (0.1 - 100 nM) were added to HD3 cells ( $1 \times 10^6$ /ml in MEM (Eagle's Minimum Medium) + 1% BSA) and incubated for 3 hours. Fig. 13 shows that both the conjugates and also the complexes bind to HD3 cells in such a way that saturation occurs. The apparent binding constants calculated from these data amounted to 22 nM for TfpL and 43 nM for TfpL-DNA complexes. Although somewhat higher, these values correspond relatively well to those obtained for native transferrin, which were found to be 15 nM (Killisch).

In order to monitor the uptake of TfpL-DNA complexes into intracellular vesicles, first of all HD3 cells were incubated at 37°C for 18 hours with transferrin-free differentiating medium. After the addition of FITC transferrin or TfpL conjugates (labelled with FITC at the polylysine group, and complexed with DNA in some experiments), the cells were incubated for a further 18 hours. The cells were cytocentrifuged, fixed with a mixture of 3.7% formaldehyde and 0.02% glutaraldehyde, washed with PBS, embedded in Mowiol 4.88 and examined under a Zeiss Axiophot Fluorescence Microscope. The control used consisted of FITC-labelled goat anti-mouse antibodies (0.1 mg/ml) (see Example 5). For quantitative determination of FITC-Tf, FITC-TfpL and FITC-TfpL/DNA, the cells were incubated for 6 hours with the preparation in question (Tf: 40 µg/ml; TfpL270: 50 µg/ml; TfpL270 plus pB-SK-DNA (Promega Biotech, prepared by Triton-X lysis, CsCl/EtBr equilibrium density gradient centrifugation, decolorising with 1-butanol and dialysis against 100 mM Tris/HCl pH 7.5, 1 mM EDTA): 50 µg/ml or 16 µg/ml; binding buffer), washed 3 times in cold PBS/BSA and subjected to quantitative FACS analysis in a Becton-Dickinson (BD) FACScan.

Fig. 14 shows that both with TfpL and with Tf

LDNA, all the cells have a relative fluorescence increased more than 10 times, indicating that the conjugates or complexes have been taken up by more than 95% of the cells (Tf:.....; TfpL: . . . ; TfpL/DNA: \_\_\_\_; binding buffer: \_\_\_\_\_).

Example 12

Expression of DNA absorbed into the cell by means of TfpL

After it had been established in the preceding Examples that the gene transfer with TfpL is not harmful to cell growth, the activity of TfpL-DNA complexes which were used themselves for a longer period of time was tested (the DNA used was plasmid DNA containing the luciferase gene, as described in Example 9). In this test, the same concentration of HD3 cells was incubated for 1 to 4 days with or without daily supplementing with TfpL-DNA complexes.

At various intervals of time, aliquots were investigated for lucerifase enzyme activity, as described in Example 9. In the cultures with repeated addition of the complexes, a relatively high level of luciferase gene expression was measured (100,000 to 200,000 light units per  $10^7$  cells), which remain substantially constant throughout the entire observation period. During this period, no cytotoxic effects were observed. If cells were charged with the complexes only once, the luciferase activity decreased between the 2nd and 4th days by a factor of 10 to 20. These results show that in spite of the obviously transient expression of the luciferase gene, introduced into the cell with the aid of the conjugates according to the invention, a constantly high expression of the introduced genes can be maintained by repeated addition.

Example 13

In order to establish how large a proportion of cells actually express plasmid DNA introduced by transferrin infection, HD3 cells were incubated with TfpL/DNA complexes as described in the preceding Examples. pRSV- $\beta$ Gal plasmid DNA (—) was used as the DNA. The expression of this reporter gene was then investigated in individual cells by FACS analysis (Nolan et al., 1986). TfpL-pB-SK-DNA (...) was used as the control. The fluorescence  $\beta$ Gal substrate FDG (fluorescene-Di- $\beta$ -d-galactopyranoside) was introduced by osmotic shock and the distribution of cells containing fluorescene which was released from FDG by the  $\beta$ Gal enzyme activity was investigated. Uniform distribution of fluorescene-containing cells leads to the conclusion that a large proportion of cells express the  $\beta$ Gal reporter gene. The results of these tests are shown in Fig. 15.

2012

37

Table A  
Transporting of Polylysine-transferrin into erythroblasts

Batch	Medium	Transferrin-polylysine	Vesicle fluorescence		Viability ( $^{3}\text{H}$ Td R-incorporation)
			24 h	48 h	
1	2 ml	without addition	<1 %	<1 %	140.000 cpm
2	2 ml	145 $\mu\text{l}$ H <sub>2</sub> O	<1 %	<1 %	126.000 cpm
3	2 ml	145 $\mu\text{l}$ TfPL Fr1	>90 %++	>90 %++	137.000 cpm
4	2 ml	145 $\mu\text{l}$ TfPL Fr2	>90 %+++	>90 %+++	161.000 cpm
5	2 ml	145 $\mu\text{l}$ TfPL Fr3	>90 %+++	>90 %+++	153.000 cpm
6	2 ml	145 $\mu\text{l}$ TfPL Fr4	ca. 80 %+++	>90 %+++	151.000 cpm
7	2 ml	145 $\mu\text{l}$ TfPL Fr5	ca. 60 %+++	>90 %+++	153.000 cpm
8	2 ml	145 $\mu\text{l}$ TfPL Fr6	ca. 40 %+++	>90 %+++	165.000 cpm

++ and +++ indicate the relative intensity of the vesicle fluorescence

Table B  
 Polylysine-transferrin can functionally replace normal transferrin in the stimulation  
 of the in vitro-induced maturation of erythroblasts

No	Medium	Addition <sup>b</sup>	No of cells (x 10 <sup>6</sup> /ml)	Haemoglobin	Degree of maturity % reticulocytes
			24 h      48 h	24 h      48 h	72 h
1	2 ml	Fe-transferrin	3.28	4.38	1.38      2.74 >80 %
2	2 ml	-	2.62	2.56	0.35      0.23 <1 %
3	2 ml	H <sub>2</sub> O	2.60	2.42	0.30      0.13 <1 %
4	2 ml	TfpL Fr1	3.70	4.44	1.36      2.69 >80 %
5	2 ml	TfpL Fr2	3.56	4.24	1.16      2.51 n.b.a
6	2 ml	TfpL Fr3	3.72	4.54	1.58      2.54 >80%
7	2 ml	TfpL Fr4	3.48	4.56	1.57      2.55 n.b.
8	2 ml	TfpL Fr5	3.36	4.26	1.41      2.47 n.b.
9	2 ml	TfpL Fr6	3.58	4.4	1.14      1.93 60-65 %

a: not determined

b: Fe-transferrin, 200 µg in 13 µl; TfpL-fraction 200 µg in 130 µl; H<sub>2</sub>O, 130 µl

2012311

Table C

Test	Transferrin	Medium-Addition Transferrin Conjugate	DNA	Cell number (x10 <sup>6</sup> /ml)	Hb (E <sup>492</sup> )	Differentiation parameter		
						% desint.	Cells	LR+E
1	-	-	-	2.56	0.259	56	<1	44
	-	-	-	3.72	1.997	3	73	1
	Tfp190	-	-	3.67	1.105	5	54	8
	Tfp1270	-	-	3.30	1.366	11	60	4
2	-	-	PRSVLuc	1.24	0.28	n.n.		
	-	-	PRSVLuc	5.22	2.459	n.n.		
	Tfp190	-	PRSVLuc	4.46	2.265	n.n.		
	-	-	-	2.1	0.222	79	<1	21
3	-	-	-	2.55	1.369	6	72	0
	-	-	-	2.64	1.016	10	56	7
	Tfp190	-	-	2.76	1.055	9	72	4
	Tf-Prot	-	-	-	-	-	-	-

Table D  
Maturation (haemoglobin content) of v-erbB - transformed erythroblasts which have absorbed  
v-erbB - ribozyme DNA

No	DNA		Transferrin	Haemoglobin content (% positive of the acetic benzidine staining)		
Type	MW	Amount	Type	Amount	14 h	62 h
1	erb-cut	13	2x10 <sup>5</sup>	1 µg	Tf	10 µg
2	erb-cut	13			TfpL Fr5	10 µg
3	erb-cut	53	2x10 <sup>5</sup>	1 µg	Tf	10 µg
4	erb-cut	53			TfpL Fr5	10 µg
5	Vector without ribozyme		2x10 <sup>6</sup>	10 µg	Tf	100 µg
6	Vector without ribozyme			10 µg	TfpL Fr5	100 µg
7	erb-cut	13 + 53 s.o.	0.5+0.5 µg	Tf	10 µg	<1
8	erb-cut	13 + 53	0.5+0.5 µg	TfpL Fr5	10 µg	<1

a: more than 200 cells were counted out for each measurement, values + or - standard deviation

b: No. of independent measurements

Claims

1. A protein-polycation conjugate comprising a polycation bound to transferrin.
2. A conjugate according to claim 1 which is capable of forming soluble complexes with substances having an affinity for polycations, said soluble complexes being capable of being absorbed into a cell by receptor-mediated endocytosis.
3. A conjugate according to claim 1 or claim 2, wherein the polycation is an optionally modified protamine.
4. A conjugate according to claim 1 or claim 2, wherein the polycation is an optionally modified histone.
5. A conjugate according to claim 1 or claim 2, wherein the polycation is a synthetic homologous or heterologous polypeptide.
6. A conjugate according to claim 5, wherein that the polypeptide is polylysine.
7. A conjugate according to any one of claims 1 to 6, comprising a polycation containing about 20 to 500 positive charges.
8. A conjugate according to any one of claims 1 to 7, having a molar ratio of transferrin to polycation of 10:1 to 1:4.
9. A protein-polycation-nucleic acid complex which is capable of being absorbed into a cell by receptor-mediated endocytosis, the protein part of the complex

being transferrin.

10. A complex according to claim 9, comprising a protein-polycation conjugate according to any one of claims 1 - 8.

11. A complex according to claim 10, comprising as nucleic acid a nucleic acid capable of specifically inhibiting genes or RNA function.

12. A complex according to claim 11, comprising a nucleic acid capable of inhibiting viral nucleic acid.

13. A complex according to claim 11, comprising a nucleic acid capable of inhibiting oncogenes or other key genes which control cell growth and/or differentiation.

14. A complex according to any one of claims 11 to 13, comprising as nucleic acid a ribozyme, or the gene coding for it.

15. A complex according to claim 14 comprising a ribozyme with a carrier RNA or a gene coding for the ribozyme together with the carrier RNA.

16. A complex according to claim 15, comprising as nucleic acid a genetic unit consisting of a tRNA gene as carrier gene and a ribozyme gene arranged inside the tRNA gene.

17. A complex according to one of claims 9 to 13, comprising as nucleic acid an optionally modified antisense-oligonucleotide, or in the case of an RNA oligonucleotide, the gene coding therefor.

18. A complex according to claim 17, comprising an optionally modified antisense-oligonucleotide with a carrier nucleic acid, or in the case of an RNA oligonucleotide, a gene coding for the RNA oligonucleotide together with a carrier RNA.
19. A process for introducing nucleic acids into human or animal cells by receptor-mediated endocytosis, comprising forming a complex from a transferrin-polycation conjugate according to any one of claims 1 to 8 and a nucleic acid or acids and bringing the cells into contact with said complex.
20. A process according to claim 19 wherein the complex is soluble under physiological conditions.
21. A pharmaceutical composition comprising one or more complexes according to any one of claims 8 to 15.
22. A protein-polycation conjugate according to claim 1 substantially as herein described.
23. A complex according to claim 10 substantially as herein described.
24. A process according to claim 19 substantially as herein described.
25. A pharmaceutical composition according to claim 21 substantially as herein described.
26. Each and every novel product, process and method described herein.

Etherstonhaugh & Co.  
Ottawa, Canada  
Patent Agents

2012311

X

Abstract

New Protein-polycation Conjugates

The invention relates to a system for transporting nucleic acids into the cell, which is effected by receptor-mediated endocytosis. Using a transferrin-polycation conjugate, a complex can be formed with the polyanionic nucleic acid. This complex is bound to the transferrin receptor, which is highly regulated in growing cells, and absorbed into the cell. Suitable nucleic acids include those which inhibit specific genes or the RNA function, such as antisense oligonucleotides or ribozymes or the genes coding for them. The invention further relates to a process for introducing nucleic acids into the cells, transferrin-polycation/nucleic acid complexes and pharmaceutical preparations containing them.

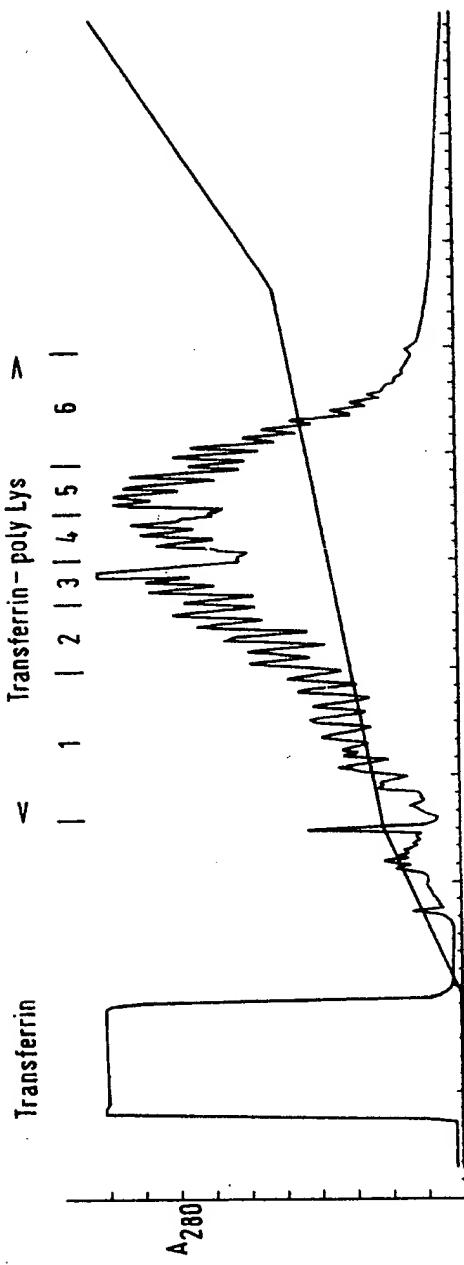


FIG. 1.

Patent Agents  
Fetherstonhaugh & Co.

(B)

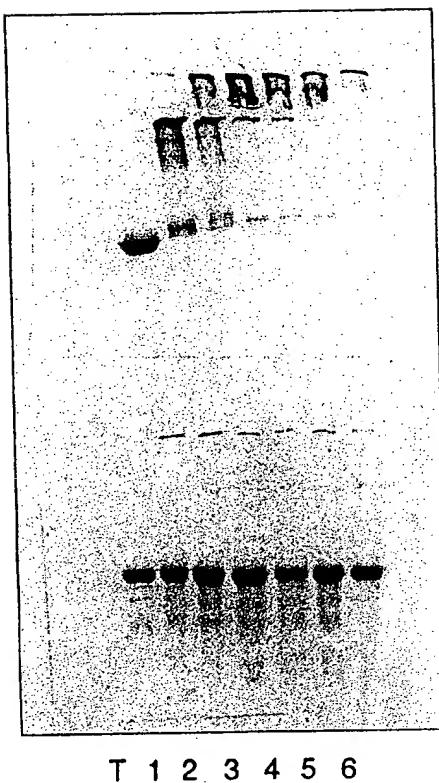


FIG. 2.

(A)

T 1 2 3 4 5 6

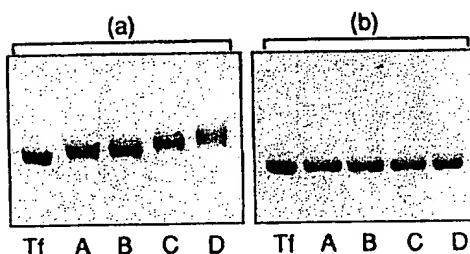


FIG. 3.

Patent Agents  
Fetherstonhaugh & Co.

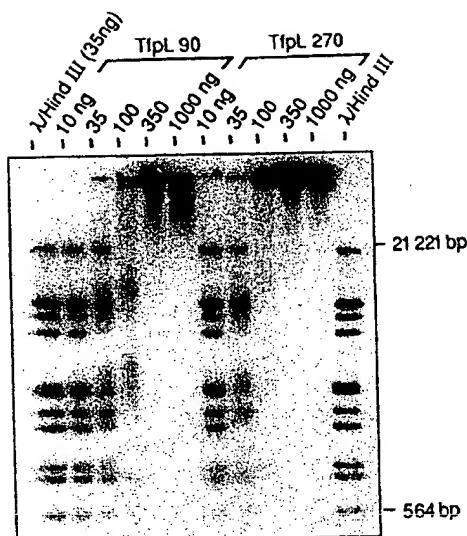


FIG. 4.

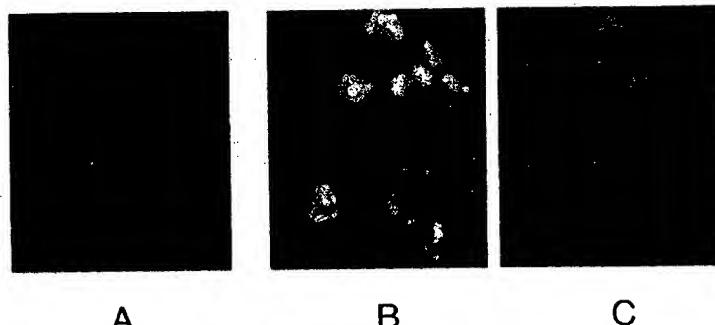


FIG. 5.

Patent Agents  
Fetherstonhaugh & Co.

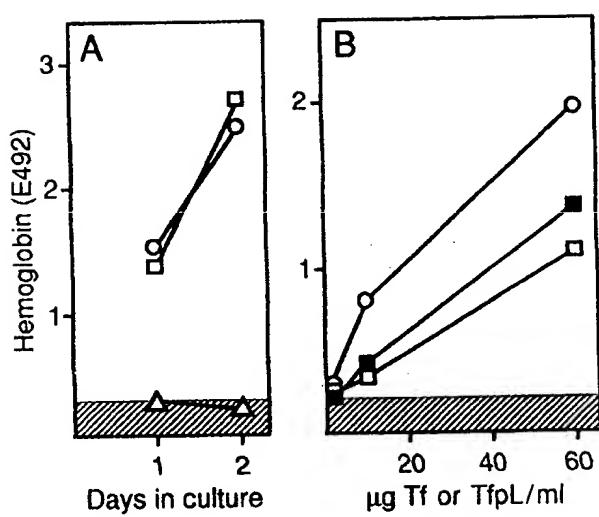
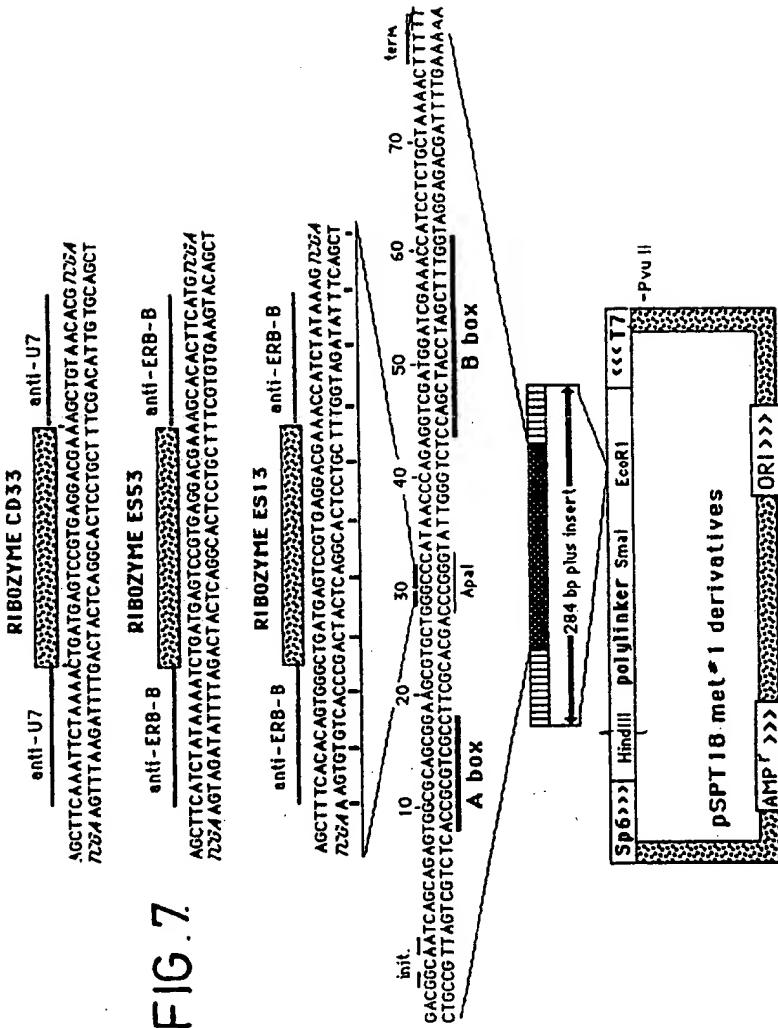


FIG. 6.

Patent Agents  
Fetherstonhaugh & Co

FIG. 7.



Patent Agents  
Fetherstonhaugh & Co.

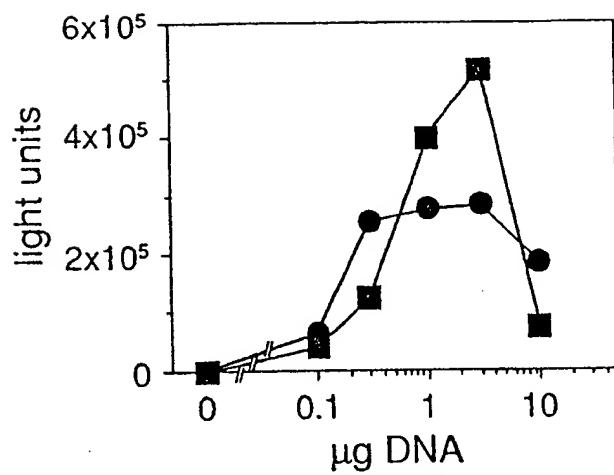


FIG. 8.

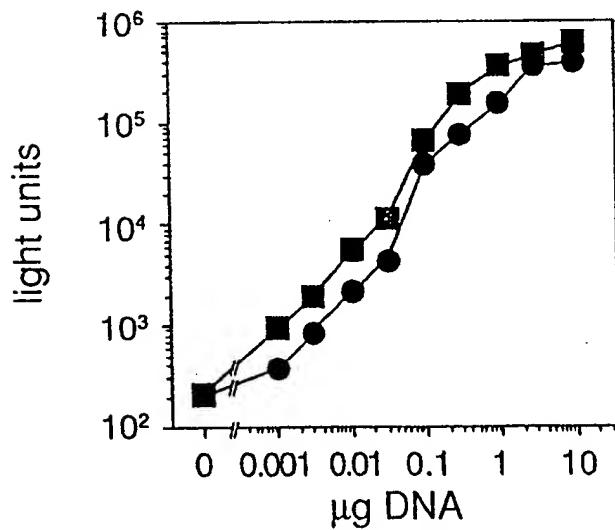


FIG. 9.

Patent Agents  
Fetherstonhaugh & Co.

FIG. 10A.

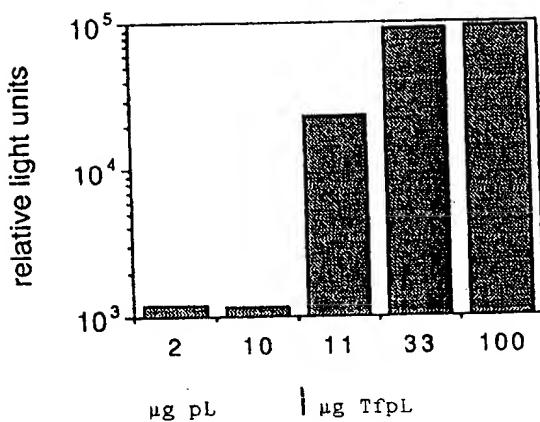
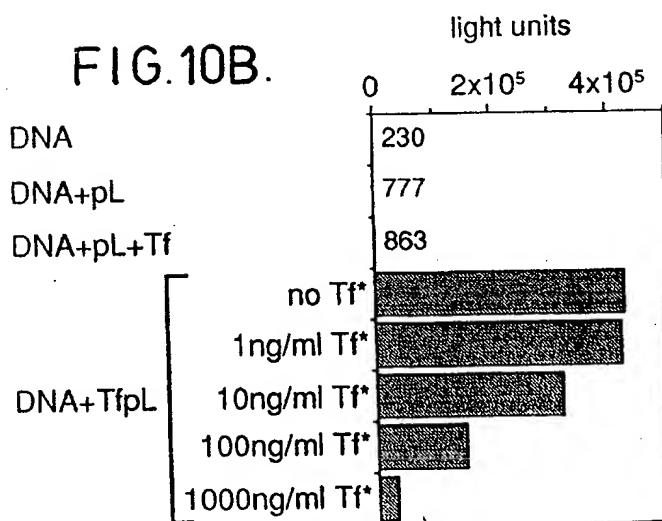


FIG. 10B.



Patent Agents  
Fetherstonhaugh & Co.

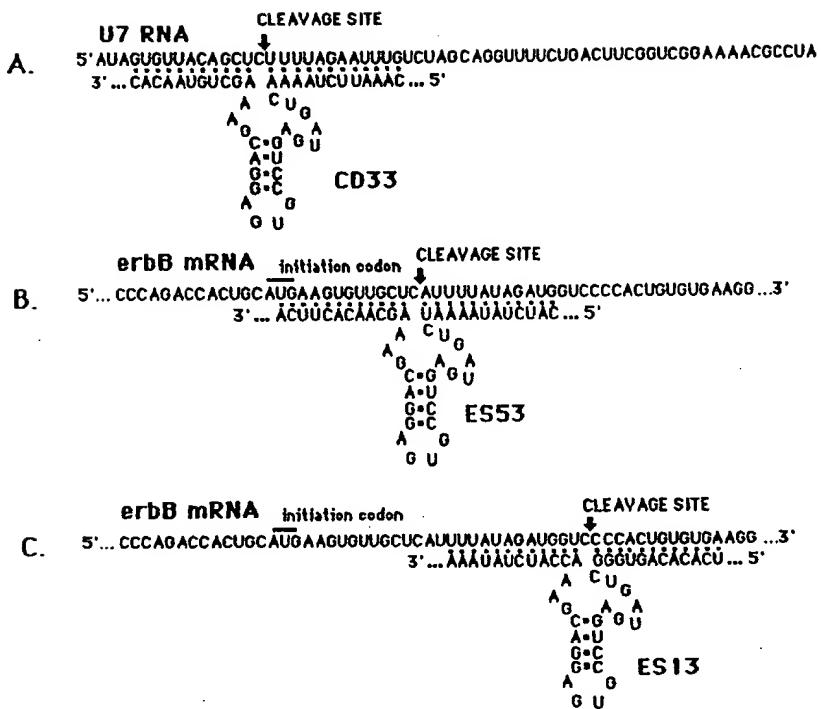


FIG. 11.

Patent Agents  
 Fetherstonhaugh & Co.

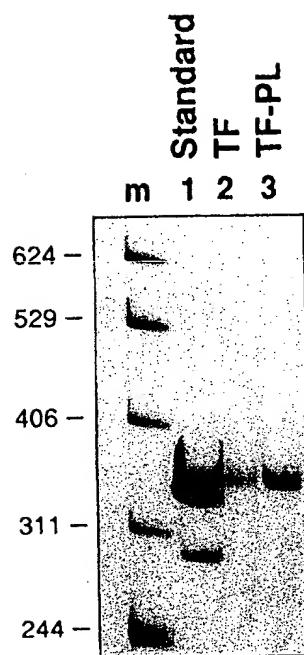


FIG. 12.

Patent Agents  
Fetherstonhaugh & Co.

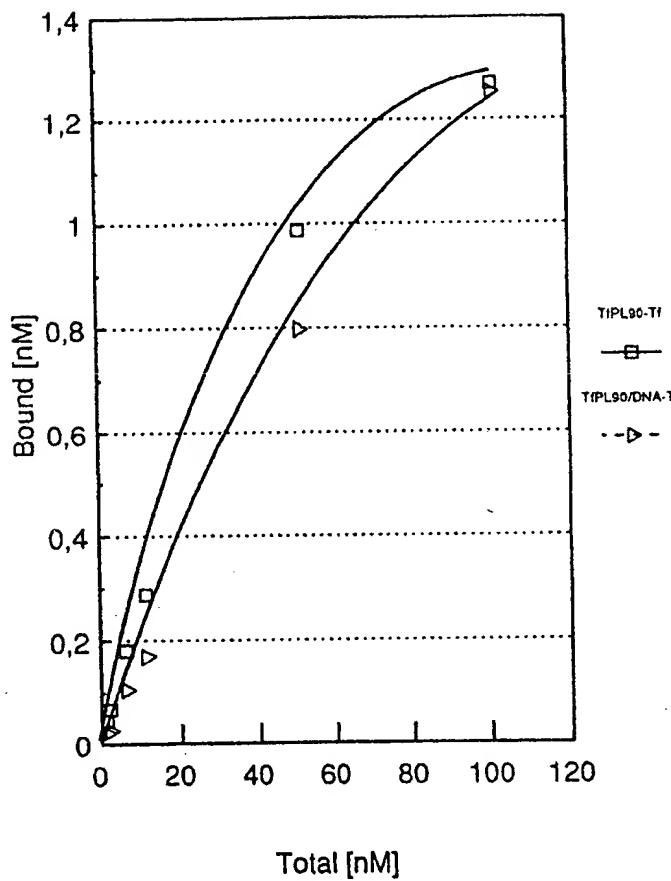


FIG.13.

Patent Agents  
Fetherstonhaugh & Co.

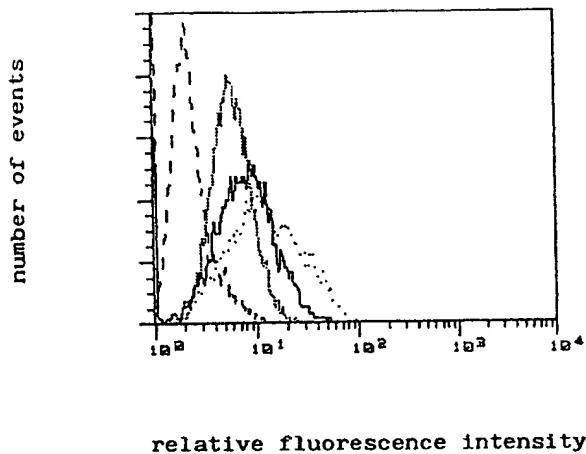


FIG. 14.

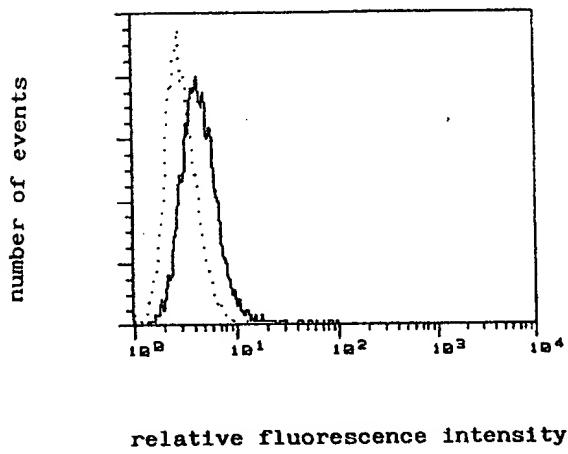


FIG. 15.

Patent Agents  
Fetherstonhaugh & Co.